Euglena gracilis Chromatin: Comparison of Effects of Zinc, Iron, Magnesium, or Manganese Deficiency and Cold Shock†

K. H. Falchuk, P. R. Gordon,‡ A. Stankiewicz, K. L. Hilt, and B. L. Vallee*
Center for Biochemical and Biophysical Sciences and Medicine and Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts 02115
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ABSTRACT: The effects induced by Fe, Mn, or Mg deficiency or cold shock on the DNA content and histones of *Euglena gracilis* have been examined and compared to those produced by Zn deficiency. The DNA content of the stationary-phase organisms used as controls is 2.1 $\mu g/10^6$ cells. The DNA of stationary-phase iron-deficient (−Fe), magnesium-deficient (−Mg), manganese-deficient (−Mn), zinc-deficient (−Zn), and cold-shocked (CS) cells is increased to 3.0, 4.6, 6.2, 3.8, and 3.8 $\mu g/10^6$ cells, respectively. The electrophoretic mobilities of proteins solubilized with 0.4 N H_2SO_4 from CS, −Fe, −Mg, and −Mn cells are nearly identical and are characteristic of the five histone classes, H_1, H_2A, H_2B, H_3, and H_4. In contrast, no histones are found in the equivalent acid extract from −Zn cells. The effect of micrococcal nuclease on chromatin from control, CS, and −Zn cells was examined. The chromatin of CS cells is 1.2-fold while that from −Zn cells is 10–30-fold more resistant to micrococcal nuclease digestion than is the chromatin of control cells. Thus, the chromatin of cells grown in Zn-deficient conditions differs markedly from that of organisms cultured in media deficient in Fe, Mn, or Mg or exposed to cold shock.

Nutritional or hormonal deficiencies or changes in ambient temperature of both prokaryotic and eukaryotic cells result in (1) proliferative arrest, (2) decreases in de novo synthesis of DNA and RNA, (3) formation and/or repression of specific proteins, (4) increases in protein breakdown, and (5) decreases in carbohydrate utilization (Ashburner & Bonner, 1979; Moran et al., 1979; McAlister & Finkelstein, 1980; Yamamiri et al., 1980; Vallee & Falchuk, 1981; Cozzone, 1981; Schlessinger et al., 1982a,b; Heikkila et al., 1982).

Euglena gracilis responds to Zn deficiency in a variety of ways. Some of these responses are among those listed above, but others have not been reported in any other organism as a consequence of either nutritional deficiencies or environmental perturbations (Price & Vallee, 1962; Wacker, 1962; Falchuk et al., 1975a–c; Crossley et al., 1982; Stankiewicz et al., 1983; Mazus et al., 1984). Zn deficiency arrests the cell cycle at S/G_2, results in the absence of histones in acid extracts of chromatin, causes the appearance of 2000–3000-dalton polypeptides, and increases the resistance of chromatin to micrococcal nuclease digestion (Falchuk et al., 1975c; Stankiewicz et al., 1983; Mazus et al., 1984). It is important, therefore, to determine which of these changes are due specifically to zinc deficiency and which are common responses to altering, in any way, the external environment of *E. gracilis*.

This report describes a number of the responses of *E. gracilis* to deficiencies of the essential metals zinc, iron, manganese, or magnesium or to cold shock. The results demonstrate that the effects of Zn deficiency on chromatin composition and structure are characteristic and specific.

Materials and Methods

Euglena gracilis, strain Z, was grown in the dark at 20 °C in control and Zn-, Fe-, Mg-, or Mn-deficient media as previously described (Falchuk et al., 1975a; K. L. Hilt et al., submitted for publication). Cold-shocked (CS) cells were obtained by transferring log-phase control cells to a 4 °C environment. After 25 days, these cells either were used for analysis or were restored to 20 °C to determine whether or not the effects of cold shock were reversible. The proliferative response of each of these cultures was examined by performing cell counts as previously described (Falchuk et al., 1975).

The DNA content of stationary-phase control, −Fe, −Mn, −Mg, −Zn, and CS cells was measured by using 4′,6-diamidino-2-phenylindole (Kapuscinski & Skoczylas, 1977). Calf thymus DNA was used as a standard. Cells were harvested by centrifugation at 1000*g* and then washed 3 times with approximately 200 mL of deionized water, extracted with 90% (v/v) acetone at 4 °C, and sonicated 30 s (50% duty cycle, output 5) using a Branson cell sonifier (Model 350).

Acid-soluble proteins from each cell type were isolated as previously described (Mazus et al., 1984). Cells were suspended in 10 volumes of 0.075 M NaCl, 0.024 M ethylenediaminetetraacetic acid (EDTA), and 0.05 M sodium bisulfite, pH 3.4. Suspensions were frozen and thawed 3 times, and then 4 N H_2SO_4 was added slowly to a final concentration of 0.4 N. Mixtures then were sonicated and stirred for 4 h at 4 °C. Precipitates were collected by centrifugation and the pellets reextracted 3 times with 25-mL portions of 0.4 N H_2SO_4. Supernatants were combined and proteins precipitated by the addition of 5 volumes of 95% (v/v) ethanol at −20 °C. Precipitated proteins were collected after 48 h by centrifugation, washed in cold 95% (v/v) ethanol, and then dissolved in 0.5 mM phenylmethanesulfonyl fluoride (PMSF) in H_2O. Samples then were adjusted to 25% (w/v) trichloroacetic acid. The resultant precipitates were centrifuged and the pellets dissolved in 0.5 mM PMSF in H_2O. Any soluble material in the supernatant was precipitated by the addition of 5 volumes of acetone and the resulting precipitate dissolved in 0.5 mM PMSF in H_2O.

Chromatin was prepared from control, −Zn, or CS organisms by suspending 6 g of cells in 10 mL of buffer A [15 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 7.5, 60 mM KCl, 15 mM NaCl, 15 mM β-mercaptoethanol, 0.15 mM spermine, 0.5 mM spermidine, 2 mM

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‡ Present address: Human Nutrition Research Center on Aging, Tufts University, Boston, MA 02111.

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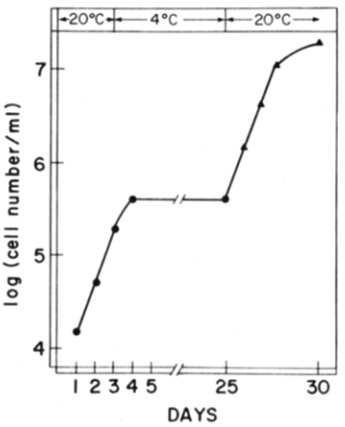

FIGURE 1: Effect of cold shock (CS) on *E. gracilis* growth. Cells cultured at 20 °C are allowed to grow into early log phase for 3 days and then are transferred to a 4 °C environment. After 25 days, cells are brought back to a 20 °C environment, and cell division resumes.

EDTA, 1 mM PMSF, and 0.34 M sucrose]. Suspensions were frozen and thawed 3 times and then sonicated for 15-s intervals for up to 8 times. The extent of cell breakage was monitored by light microscopy. Suspensions were then layered on buffer B [buffer A containing 0.2 mM EDTA and 40% (w/v) sucrose] and centrifuged 15 min at 2000*g*. The resultant pellet, composed of nuclei and paramylon, was resuspended in buffer C (buffer A but containing 0.4 mM EDTA). This procedure was repeated 3–5 times to separate chromatin from nuclear membranes.

Acid-soluble proteins also were extracted directly from chromatin of control, CS, or −Zn cells. Chromatin was mixed with 20 volumes of 0.4 N H_2SO_4 at 4 °C for up to 70 h and then centrifuged for 20 min at 8000*g*. Five volumes of 95% ethanol was added to the resultant supernatant and the mixture incubated at −20 °C for 48 h. The resultant precipitate was collected by centrifugation and the supernatant precipitated again with ethanol. Pellets were dissolved in 0.5 mL of sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) electrophoresis sample buffer (0.05 M Tris-HCl, pH 6.8, 2.5 mM β-mercaptoethanol, and 0.1% SDS) and then dialyzed 3 × 24 h against 60 volumes of the same buffer adjusted to 1.2% β-mercaptoethanol and 10% (w/v) glycerol.

Acid-extracted proteins from whole cells or chromatin were separated on 15% SDS–polyacrylamide gels (Laemmli, 1970). Electrophoresis was performed for 3 h at 20 mA. Gels were fixed for 1 h in H_2O/formaldehyde (6:1), stained with 0.1% Coomassie Blue, and destained in H_2O/methyl alcohol/formaldehyde (75:25:1).

The extent to which micrococcal nuclease digests chromatin was determined as described (Stankiewicz et al., 1983). Briefly, chromatin pellets were washed 3 times with and suspended in buffer D (buffer A, but without EDTA). $MgCl_2$ and $CaCl_2$ were added to final concentrations of 1 mM. Chromatin (400 μg of DNA) then was incubated with 100 units of micrococcal nuclease at room temperature. Nuclease digestion was terminated by addition of EDTA to samples to a final concentration of 20 mM. Samples were cooled on ice and then centrifuged for 10 min at 18000*g*. The amount of DNA digested was determined by measurement of the A_{260} of the supernatant, acid-soluble fractions.

RESULTS

Stationary-phase *E. gracilis* cells cultured in control media at 20 °C enter log-phase growth after an initial lag period of 1–2 days. When log-phase cells are transferred to a 4 °C environment on day 3, they divide once and then cease to proliferate by day 4 (Figure 1). The morphological appearance of stationary-phase (CS) cells is identical with that reported for −Zn *E. gracilis* (Falchuk et al., 1975a). The growth arrest of CS cells is completely reversible even after 25 days. When these cells are returned to 20 °C, the onset of cell division is rapid. It occurs with a very short lag phase (only 2–3 h) and achieves a rate of division characteristic of control log-phase cells. When reversed CS cells reach plateau growth, the final population density attained is typical of control cultures grown at 20 °C.

Table I: Effect of Growth Conditions on *E. gracilis* DNA Content

cell type	DNA[a] (μg/10^6 cells)	cell type	DNA[a] (μg/10^6 cells)
control	2.1 ● 0.3	−Zn	3.8 ± 0.6
CS	3.8 ● 0.4	−Mg	4.6 ± 0.4
−Fe	3.0 ± 0.2	−Mn	6.2 ± 0.6

[a] DNA values are for stationary-phase cells.

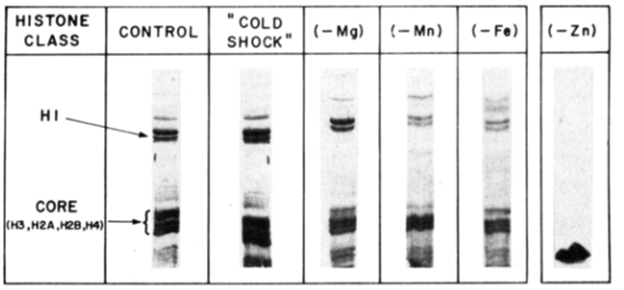

FIGURE 2: Electrophoretic mobilities of sulfuric acid soluble proteins from *E. gracilis* cells. Proteins from control, CS, −Fe, −Mg, or −Mn organisms are obtained by precipitating the sulfuric acid extracts with 25% (w/v) trichloroacetic acid (TCA). The material from (−Zn) cells differs in that it is completely soluble in 25% (w/v) TCA and is obtained by precipitating with acetone. The arrows identify bands which correspond to that of *E. gracilis* histone H_1 and the core histones H_2A, H_2B, H_3, and H_4. No histones are found in the −Zn extract. The single band observed in −Zn samples is the predominant material present in the sulfuric acid extract from these cells.

The growth characteristics of −Zn, −Fe, −Mn, or −Mg cells have been described elsewhere (Falchuk et al., 1975a; K. L. Hilt et al., submitted for publication). Importantly, under the conditions used, the population densities of stationary-phase cells in all these deficiencies are approximately 10% of control cultures. In each case, the growth arrest is reversed following addition of the omitted metal.

The amount of DNA of stationary-phase cells cultured under the various experimental growth conditions is shown in Table I. In all cases, total DNA is increased over that in control cells.

The proteins of control, −Fe, −Mg, −Mn, or CS cells solubilized by sulfuric acid and then precipitated by trichloroacetic acid separate on 15% SDS–PAGE gels into five major and several minor components (Figure 2). The electrophoretic behavior of the major components is the same as that of purified *E. gracilis* histones (Mazus et al., 1984). Furthermore, the mobilities of the proteins are identical with those extracted from chromatin of control or CS cells (not shown). Histones are entirely absent, however, from similarly prepared material from −Zn cells. Instead, the sulfuric acid extract from −Zn cells in composed primarily of 2000–3000-dalton polypeptides (Figure 2) as previously reported (Stankiewicz et al., 1983; Mazus et al., 1984). These polypeptides are soluble in 25% trichloroacetic acid and precipitate on addition of acetone.

The ability of micrococcal nuclease to digest chromatin from CS or −Zn cells has been compared (Figure 3). Chromatin obtained from CS cells is only slightly, i.e., 1.2-fold, more resistant to digestion than chromatin from control cells. This

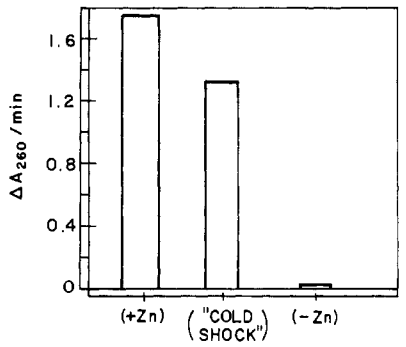

FIGURE 3: Micrococcal nuclease digestion of chromatin from control, CS, or −Zn cultures. The amount of DNA solubilized from each of these chromatin preparations is compared.

is in contrast with chromatin from −Zn cells which is 10–30-fold more resistant, confirming previous observations (Stankiewicz et al., 1983).

Discussion

The components of chromatin, in particular DNA and histones, of stationary-phase control, CS, −Zn, −Fe, −Mn, or −Mg *E. gracilis* cells have been examined in order to determine whether the changes in these components induced by Zn deficiency are specific for this latter environmental perturbation. The extent of proliferative arrest in cultures of CS, −Fe, −Mn, or −Mg cells utilized for these studies was identical with that normally produced by zinc deprivation (Falchuk et al., 1975a; K. L. Hilt et al., submitted for publication). This equality was considered necessary in order to make meaningful the comparison of the compositional changes induced by each growth condition.

The DNA content of cells from each of these cultures is greater than that in controls, although by different amounts (Table I). These DNA values, together with knowledge of the morphological changes caused by each of these culture conditions (Falchuk et al., 1975a; K. L. Hilt et al., submitted for publication), allow for identification of the phase of the cell cycle blocked by each of the environmental perturbations. The quantity of DNA present in −Fe cells is typical of cells known to be in early to mid S phase of the cell cycle (Falchuk et al., 1975c), a finding which identifies this stage as the one in which these cells are blocked. The DNA content of CS organisms is typical of late S/G_2-blocked *E. gracilis* and is similar to that of −Zn cells (Falchuk et al., 1975c). This similarity in the cell cycle stage blocked by −Zn and CS cells has not previously been reported. The basis for this particular block is unknown and will require additional experimental work to define the processes affected by these two different environmental stresses.

Two other cell types, −Mg and −Mn, have DNA contents even higher than that of G_2-phase control cells. Such a finding is characteristic of heteroploid organisms. Like other eukaryotes, *E. gracilis* normally is haploid except during and following the S phase of the cell cycle. In addition, we have previously observed heteroploidy in −Zn *E. gracilis* cells incubated with Cd (Falchuk et al., 1975b). Morphological analysis of −Mn or −Mg cells reveals that heteroploidy in these cells results from accumulation of two or more nuclei within each organism (K. L. Hilt et al., submitted for publication). Both cell types appear unable to terminate cytokinesis so they occur as multiple daughter cells still attached to each other. −Mn and −Mg organisms differ, however, in the number of nuclei per cell. −Mg cells have only one nucleus in each daughter cell structure, or lobe, while −Mn cells often have several nuclei in each lobe. In −Mn cells, therefore, karyokinesis as well as cytokinesis is affected.

These results indicate that blocks and alterations in the cell cycle of *E. gracilis* are different for each metal deficiency. This argues against the existence of a common process which is turned on or off when a metal becomes limiting. In the latter case, the block underlying the proliferative arrest of all the cultures would be expected to occur at the same stage of the cell cycle. The occurrence of blocks at different stages suggests either unique roles for each metal in the cell cycle and/or the existence of distinct limiting processes in the cycle for each deficiency state. These possibilities have been discussed previously (Vallee & Falchuk, 1981; K. L. Hilt et al., submitted for publication).

Despite large differences in DNA content, the acid extracts from control, −Fe, −Mg, −Mn, or CS cells all contain soluble proteins whose electrophoretic behavior is typical of the five major histone classes (Figure 2). This contrasts with −Zn cells from which histones are not solubilized with this standard extraction method (Figure 2). As previously reported, the absence of histones in acid extracts is reversed upon zinc replenishment of −Zn *E. gracilis* (Mazus et al., 1984). The characterization of the proteins which remain associated with the chromatin from −Zn cells after acid treatment and their relationship to both histones and non-histone proteins present in each of the other cell types need to be determined.

It would be expected that the chromatin of cell types, such as CS, would have a structural organization whose response to micrococcal nuclease action would be similar to control cells (Stankiewicz et al., 1983). The availability of large quantities of control and (CS) cells which provide sufficient chromatin for study has allowed us to obtain data consistent with this proposition for these two cell types (Figure 3). These findings differ from those reported for Zn-deficient *E. gracilis* where the sensitivity of chromatin to micrococcal nuclease is markedly different from that of Zn-sufficient cells (Stankiewicz et al., 1983; Mazus et al., 1984).

The possible bases for the absence of histones in acid extracts of −Zn cells have been discussed previously and are being examined at present in terms of a number of mechanisms (Mazus et al., 1984). These include changes in chemical modification of histones which result in much tighter binding to DNA and prevent the solubilization of these proteins by acid, enhanced degradation of histones by specific proteases, or repression of histone genes, among others. Whatever the explanation, it is concluded that the absence of histones in acid extracts from chromatin and the increased resistance of the latter to nuclease digestion are unique and specific to Zn deficiency.

Registry No. Fe, 7439-89-6; Mn, 7439-96-5; Mg, 7439-95-4; Zn, 7440-66-6.

References

Ashburner, M., & Bonner, J. J. (1979) *Cell (Cambridge, Mass.) 17*, 241–254.

Cozzone, A. J. (1981) *Trends Biochem. Sci. (Pers. Ed.) 6*, 108–110.

Crossley, L. G., Falchuk, K. H., & Vallee, B. L. (1982) *Biochemistry 21*, 5351–5363.

Falchuk, K. H., Fawcett, D., & Vallee, B. L. (1975a) *J. Cell Sci. 17*, 57–78.

Falchuk, K. H., Fawcett, D., & Vallee, B. L. (1975b) *J. Submicrosc. Cytol. 7*, 139–152.

Falchuk, K. H., Krishan, A., & Vallee, B. L. (1975c) *Biochemistry 14*, 3439–3444.

Heikkila, J. J., Schultz, B. A., Iatron, K., & Gedamn, L. (1982) *J. Biol. Chem. 257*, 12000–12005.

Kapuscinski, J., & Skoczylas, B. (1977) *Anal. Biochem. 83*, 252–257.
Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.
Mazus, B., Falchuk, K. H., & Vallee, B. L. (1984) *Biochemistry 23*, 42–47.
McAlister, L., & Finkelstein, D. B. (1980) *Biochem. Biophys. Res. Commun. 93*, 813–824.
Moran, L., Mirault, M. E., Arrigo, A. P., Goldschmidt-Clemont, M., & Tissieres, A. (1978) *Philos. Trans. R. Soc. London, B 283*, 299–307.
Price, C. A., & Vallee, B. L. (1962) *Plant Physiol. 37*, 428–433.
Schlesinger, M. J., Ashburner, M., & Tissues, A. (1982a) *Heat Shock: From Bacteria to Man*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Schlesinger, M. J., Aliperti, G., & Kelly, P. M. (1982b) *Trends Biochem. Sci. (Pers. Ed.) 7*, 222–225.
Stankiewicz, A. J., Falchuk, K. H., & Vallee, B. L. (1983) *Biochemistry 22*, 5150–5156.
Vallee, B. L., & Falchuk, K. H. (1981) *Philos. Trans. R. Soc. London, B 294*, 185–197.
Wacker, W. E. C. (1962) *Biochemistry 1*, 859–865.
Yamamiri, J., Ito, K., Nakama, Y., & Yura, T. (1980) *Cell (Cambridge, Mass.) 22*, 825–834.

Identification of the Arylazido-β-alanyl-NAD$^+$-Modified Site in Rabbit Muscle Glyceraldehyde-3-phosphate Dehydrogenase by Microsequencing and Fast Atom Bombardment Mass Spectrometry†

Shiuan Chen,* Terry D. Lee, Kassu Legesse, and John E. Shively
Division of Immunology, Beckman Research Institute of the City of Hope, Duarte, California 91010
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ABSTRACT: We have identified the site labeled by arylazido-β-alanyl-NAD$^+$ (A3′-*O*-{3-[*N*-(4-azido-2-nitrophenyl)amino]propionyl}NAD$^+$) in rabbit muscle glyceraldehyde-3-phosphate dehydrogenase by microsequencing and fast atom bombardment mass spectrometry. This NAD$^+$ photoaffinity analogue has been previously demonstrated to modify glyceraldehyde-3-phosphate dehydrogenase in a very specific manner and probably at the active site of the enzyme [Chen, S., Davis, H., Vierra, J. R., & Guillory, R. J. (1984) *Biochem. Biophys. Stud. Proteins Nucleic Acids, Proc. Int. Symp., 3rd*, 407–425]. The label is associated exclusively with a tryptic peptide that has the sequence Ile-Val-Ser-Asn-Ala-Ser-Cys-Thr-Thr-Asn. In comparison to the amino acid sequence of glyceraldehyde-3-phosphate dehydrogenase from other species, this peptide is in a highly conserved region and is part of the active site of the enzyme. The cysteine residue at position seven was predominantly labeled and suggested to be the site modified by arylazido-β-alanyl-NAD$^+$. This cysteine residue corresponds to the Cys-149 in the pig muscle enzyme, which has been shown to be an essential residue for the enzyme activity. The present investigation clearly demonstrates that arylazido-β-alanyl-NAD$^+$ is a useful photoaffinity probe to characterize the active sites of NAD(H)-dependent enzymes.

In recent years, photoaffinity labeling techniques have been widely applied to characterize enzyme active sites. However, the usefulness of these techniques has been seriously limited for several reasons. In many investigations, difficulties were found in the characterization of the modified peptides. It is not uncommon to find that modification changes the chemical and/or physical properties of peptides and complicates the separation and structural analysis. Additionally, the attachment can be labile, and the modifying groups may be lost during the characterization process. Also, the amounts of the modified peptides may simply be too low for full characterization. Therefore, most of the studies have only been carried out as far as demonstrating the selectivity and specificity of reaction between the photoprobes and enzymes. Only a few modified active-site peptides are actually characterized. In most instances, these photoprobe-labeled peptides are identified indirectly by comparison of the amino acid composition of the modified peptides with those of the peptides with known sequences. Even in those cases where the modified peptides have been identified and the modified amino acids have been determined, there is always some uncertainty in the interpretation of the results. The question is constantly raised whether the photoprobe indeed binds to the active site, to regions near the action site, or even to regions not directly related to the activity. Because most photoprobes are synthesized by modifying the structure of the natural ligand or by adding a photoreactive group, it is reasonable to rationalize that these structural changes may have some influence on their binding to the active site. Alternatively, the probe can bind to a specific site other than the active site (e.g., allosteric site). It is also possible that the probe binds to regions resulting from a postphotolyzed interaction, if the photoactivated species has a lifetime sufficient to allow it to diffuse away from the ligand binding site before the insertion takes place. Thus, it is clear that, in order to take full advantage of the photoaffinity labeling techniques in the investigation of the active site of enzymes, it is necessary to have sensitive and direct methods to characterize the modified peptides and to understand the nature of the interaction of photoaffinity probes with the enzymes.

The recent development of gas phase microsequencing techniques allows protein/peptide sequencing at the subnanomole range (Hewick et al., 1981; Hawke et al., 1985). In addition, fast atom bombardment mass spectrometry

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* Correspondence should be addressed to this author.

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